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Hypoxia inducible factor-1 (HIF-1) is a transcription factor that governs cellular responses to reduced O_2 availability by mediating crucial homeostatic processes. The degradation of HIF-1α subunit is redox regulated. Manganese superoxide dismutase (MnSOD) is an antioxidant enzyme that can modulate cellular redox environment. Here we show that MnSOD suppresses hypoxic accumulation of HIF-1α protein. This suppression can be observed under both 1% O_2 and 4% O_2. Hypoxic induction of vascular endothelial growth factor (VEGF), a known HIF-1 targeted gene, is also suppressed by elevated MnSOD activity and its expression levels reflected the protein levels of HIF-1α. Peroxide removing enzymes located inside the mitochondria (adenoviral GPx-1 and mitochondrial-targeted catalase) inhibited HIF-1α protein accumulation in a high MnSOD expressing clone. Increasing peroxisomal/cytosolic catalase activity or inhibition of endogenous catalase by 3-amino-1,2,4-triazole had only a minor effect, suggesting that hydrogen peroxide produced inside mitochondria and not by peroxisomes could mediate the accumulation of HIF-1α protein when MnSOD activity is high. These observations demonstrate that HIF-1α accumulation is modulated not only by the levels of dioxygen but also the antioxidant enzyme MnSOD.

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Introduction

Tumor cells that have adapted to hypoxic conditions are thought to play critical roles in tumor progression (1). Among the first responses at the onset of hypoxia is an increase in the protein levels of hypoxia-inducible factor-1 (HIF-1) (2). HIF-1 responds to reduced O_2 availability by mediating crucial homeostatic processes such as angiogenesis, glycolysis, and erythropoiesis (3). As a transcription factor, HIF-1 has more than 40 target genes and the known number continues to increase. Vascular endothelial cell growth factor (VEGF) is one of the HIF-1 downstream genes (4). It is a powerful mitogenic cytokine specific for endothelial cells; its production triggers the angiogenic cascade and tumor neovascularization process (5). VEGF controls not only the onset, but also the extent and duration of these processes. Hypoxia, which is a common characteristic of solid tumors, is a stimulus leading to the induction of VEGF *via* the regulation of HIF-1 (6-8). Reactive oxygen species (ROS) such as superoxide and hydrogen peroxide are known to serve as signal transducers (9-12). Manganese superoxide dismutase (MnSOD) is a major antioxidant enzyme that is located in the mitochondrial matrix. It has been shown that the malignant phenotype is suppressed when MnSOD activity is elevated in tumor cells (13-15). It has been implicated as a tumor suppressor and as a metastasis suppressor in some tumor cell lines (16-18). MnSOD modulates the cellular redox environment by converting superoxide radical ($O_2^{\bullet-}$) to hydrogen peroxide (H_2O_2) and dioxygen. Therefore, MnSOD may affect the expression of redox-sensitive genes, including HIF-1.

Body

The objective of this research is to examine the relationship of MnSOD activity to breast tumor angiogenesis. Three Specific Aims were initially proposed:
   (1)   Determine if MnSOD overexpression alters HIF-1 activity.
   (2)   Determine if MnSOD overexpression alters VEGF expression in H-*ras* transformed human breast cancer cells.
   (3)   Determine if MnSOD overexpression alters endothelial cell proliferation and migration.

Specific Aims I and II were accomplished during the grant period. MnSOD overexpression was found to suppress HIF-1α protein accumulation with hypoxia exposure. Moreover, the induction of one of the HIF-1 target genes, VEGF, was suppressed by MnSOD-overexpression in a similar pattern as that of HIF-1α protein. However, instead of using H-*ras* transformed cells that upon reflection we thought would too specific for the purpose of the study, we used human breast cancer cell line MCF-7 wild type to test the suppressive effect of MnSOD on HIF-1 and VEGF. The five major tasks in the SOW are listed below and progress on each is summarized.

Task 1: Development of breast cancer cells with altered MnSOD expression.
----**Completed**

During the first year of the research program (2002-2003), human breast cancer cells with altered MnSOD expression was developed as proposed in Task 1. I was lucky in that a large set of MCF-7 cells that have a wide range of MnSOD activity as a result of stable plasmid transfection became available. I characterized the antioxidant profile of these cells so as to make an informed choice of which subset of these cells would be used for this study. In addition I adapted protocols to use adenoviral induction to elevate MnSOD activity in MCF-7 cells. The protocols needed to produce a range of MnSOD activity in MCF-7 cells were developed and protein expression and activities levels were verified.

Task 2: Determine if level of MnSOD activity alters HIF-1 activation in human breast cells.
----Completed

We have shown that MnSOD suppressed hypoxic accumulation of HIF-1α protein in human breast carcinoma MCF-7 cells. This suppression was biphasic depending on MnSOD activity. At low levels of MnSOD activity, HIF-1α protein accumulated under hypoxic conditions. At moderate levels of MnSOD activity (2- to 6-fold increase compared to parent cells), these accumulations were blocked. However, at higher levels of MnSOD activity (>6-fold increase), accumulation of HIF-1α protein was again observed. This biphasic modulation can be observed under both 1% O_2 and 4% O_2. (The Figure below shows the results at 1% oxygen.)
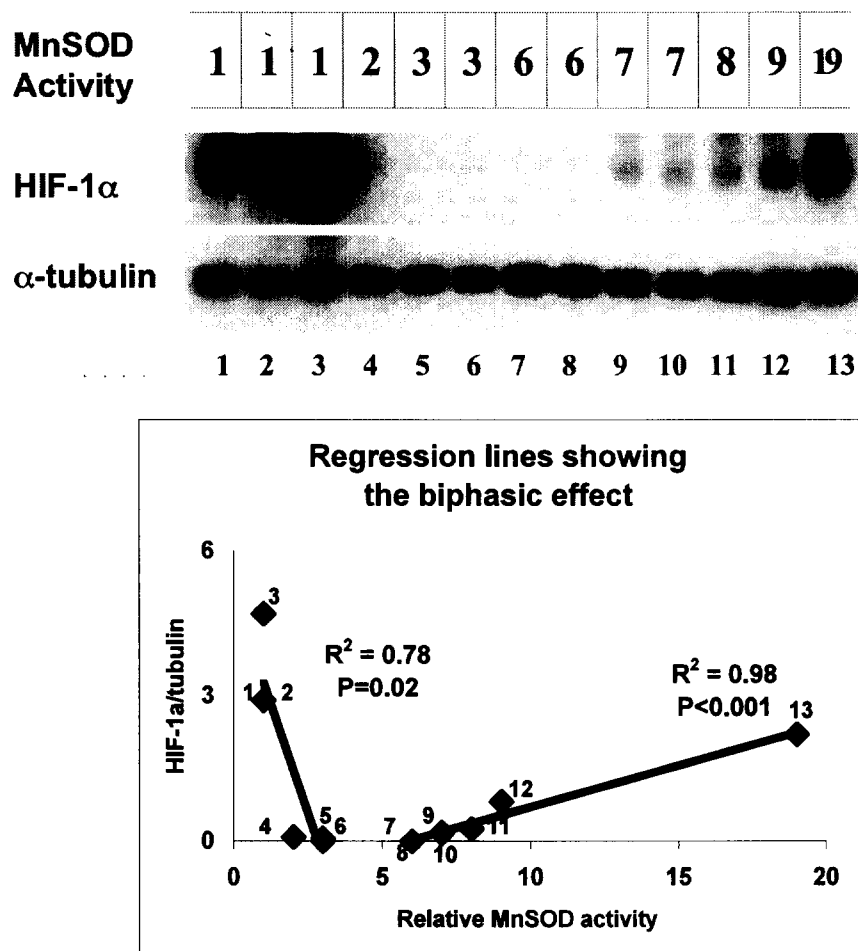
Figure. Plasmid transfection of MnSOD suppressed HIF-1α protein levels at 1% O_2.
  Top: Western blot showed HIF-1α protein expression in 13 different MnSOD stably transfected clones of MCF-7 cells after 4 h of growth in 1% O_2. The first 3 lanes were samples from: MCF-7 parent cells, Neo vector control and a clone with same activity as parental cells, respectively. The remaining 10 lanes were samples from clones with increasing MnSOD activity. The top row of numbers showed the relative MnSOD activity compared to the parental MCF-7 cells. Similar results have been obtained in a repeat experiment. α-Tubulin was used as internal control.
  Bottom: Regression analysis shows a biphasic effect. HIF-1α protein levels were quantified by densitometry and normalized to α-tubulin levels. Thirteen points are shown, some overlay. The numbers next to points correspond to the lane number in top panel.

We did parallel experiments at 4% oxygen and observed similar trends, however as might be expected at these higher levels of oxygen, HIF-1α protein levels were decreased overall compared to 1% oxygen.

In addition, we demonstrated that HIF-1α was regulated post-translationally in the above experimental settings as HIF-1α mRNA was not changed significantly with hypoxia exposure compared to the changes of protein levels. These observations demonstrate that HIF-1α accumulation can be modulated by the antioxidant enzyme MnSOD.

• Use gel shift and supershift to measure the DNA binding activity of HIF-1 transcriptional complex.
----Since the expression of one of the HIF-1 downstream gene, VEGF, was studied to verify the activity of HIF-1 under hypoxia, this subtask was substituted by the gene expression study of VEGF.

Task 3: Determine if level of MnSOD activity alters the level of VEGF expression or the level of VEGF 121 isoform in human breast cells.
----**Completed**
• Generate a riboprobe for VEGF$_{121}$.
---- VEGF primer used for RT-PCR was designed and tested.
• Subject cells to conditions known to activate VEGF expression (hypoxia).
----Routine work.
• Use western and northern blotting to measure protein and mRNA of VEGF.
----Both VEGF mRNA and protein levels were suppressed in a similar pattern as that of HIF-1α protein with the overexpression of MnSOD. RT-PCR was used to measure qualitatively mRNA levels of VEGF. VEGF Elisa assay was used to measure VEGF protein levels.

Hypoxic induction of vascular endothelial growth factor (VEGF), a known HIF-1 target gene, was also suppressed by elevated MnSOD activity and its expression levels reflected HIF-1α protein levels.
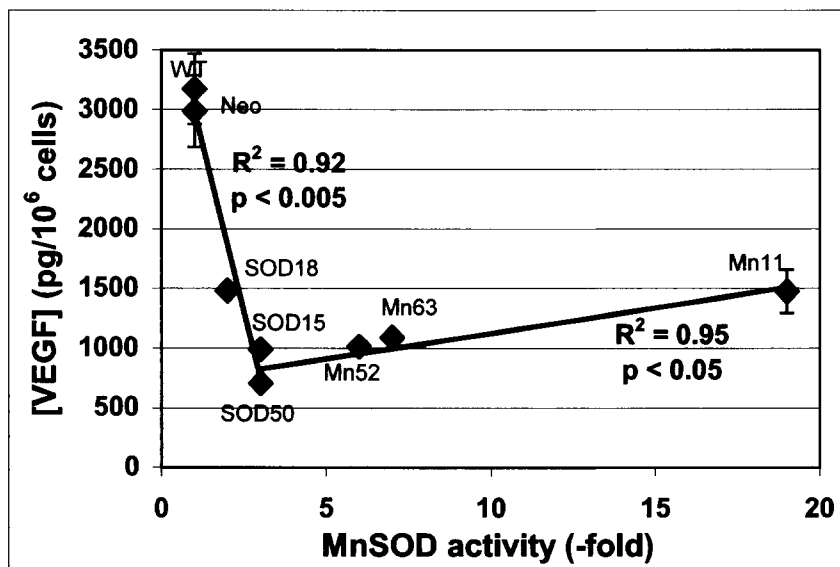

Figure. MnSOD overexpression suppressed VEGF protein secretion in MCF-7 cells. VEGF protein was measured after 12 h of exposure to hypoxia (1% O_2) in 8 distinct MCF-7 clones with various MnSOD activities. VEGF protein secretion was suppressed with increased MnSOD activity. Regression analysis shows a biphasic effect parallel to the observations with HIF-1α.

These observations demonstrate that HIF-1α accumulation can be modulated by the antioxidant enzyme MnSOD and that this results in the parallel control over the expression of a key gene controlled by HIF-1α.

Task 4: Determine if level of MnSOD activity in breast cancer cells alters proliferation and migration of nearby endothelial cells.

This task was not started. We submitted our results from tasks 1-3 for publication and the reviewers asked that we address the molecular mechanism behind our observations in much more detail. Because of the importance of this question we devoted our energies to the question: *Is superoxide or hydrogen peroxide involved in the observations summarized above?* Because MnSOD controls the steady-state level of superoxide and produces hydrogen peroxide as a result of its enzymatic action, experiments were designed to test a possible role of hydrogen peroxide in our observations.

To address this question we devised methods to alter the ability of the cells to remove hydrogen peroxide. We inhibited catalase activity and we increased catalase activity (verified using enzyme assays as well as actual measurements of hydrogen peroxide production) and observed that this modulated the expression of HIF-1α only in the Mn11 clone, i.e. the clone with the highest level of MnSOD and the highest level of hydrogen peroxide production. To provide another test, we used adenovirus transduction of glutathione peroxidase (GPx-1), another peroxide-removing enzyme and observed exactly parallel results, i.e. only the Mn11 clone was affected. These data suggest that hydrogen peroxide is only a minor player in our observations and that superoxide is the key chemical species responsible for our observations (Figure below).
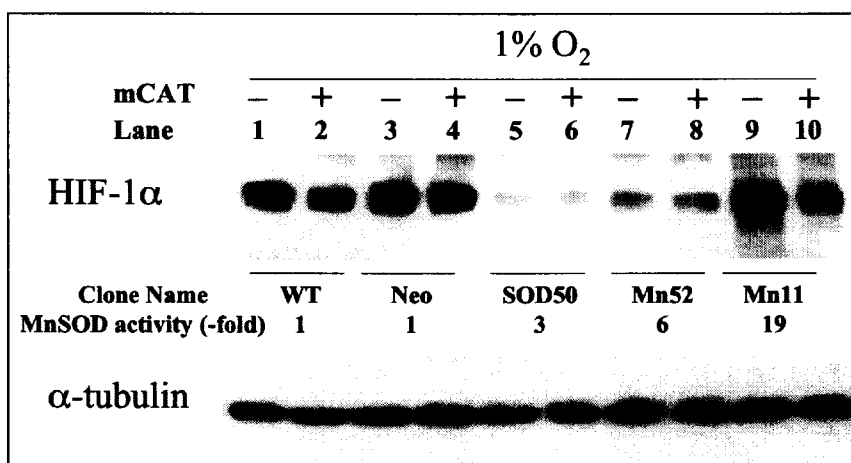
Figure. **Adenoviral transduction of mitochondrial catalase (mCAT) decreased HIF-1α protein levels in clone with high MnSOD activity.** Mitochondrial catalase (mCAT, 50 MOI) was introduced into five of the MnSOD stably transfected MCF-7 clones: MCF-7 WT and Neo, SOD50 (3-fold increase in MnSOD activity compared to MCF-7 WT), Mn52 (6-fold increase in MnSOD activity), and Mn11 (19-fold increase in MnSOD activity). Cells were incubated with adenovirus carrying mCAT for 24 h. Medium containing adenovirus was removed and fresh medium was added onto cells for another 24 h. Cells were then exposed to hypoxia for 4 h to induce HIF-1. This is a representative of three independent experiments.
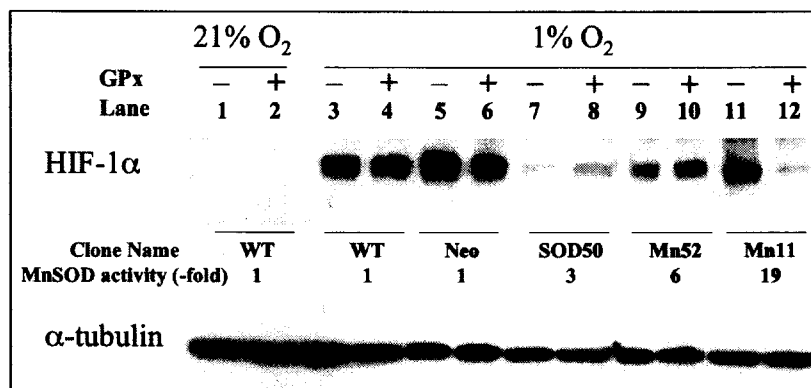
Figure. Adenoviral transduction of glutathione peroxidase-1 (GPx-1) decreased HIF-1α protein levels in clone with high MnSOD activity. Adenoviral GPx-1 (100 MOI) was introduced into five of MnSOD stably transfected MCF-7 clones: MCF-7 WT and Neo, SOD50 (3-fold increase in MnSOD activity compared to MCF-7 WT), Mn52 (6-fold increase in MnSOD activity), and Mn11 (19-fold increase in MnSOD activity). Cells were incubated with adenovirus carrying mCAT for 24 h. Medium containing adenovirus was removed and fresh medium was added onto cells for another 24 h. Cells were then exposed to hypoxia for 4 h to induce HIF-1α. This is a representative a three independent experiments.

As another approach to test the possible role of superoxide in the activation of HIF-1α, we did pilot experiments of altering copper-zinc superoxide (CuZnSOD), the cytosolic SOD. It too modulated HIF-1α protein expression. We are currently doing experiment in which we try other approaches to alter the steady-state level of superoxide in cells and determine the effect on the modulation of HIF-1α.
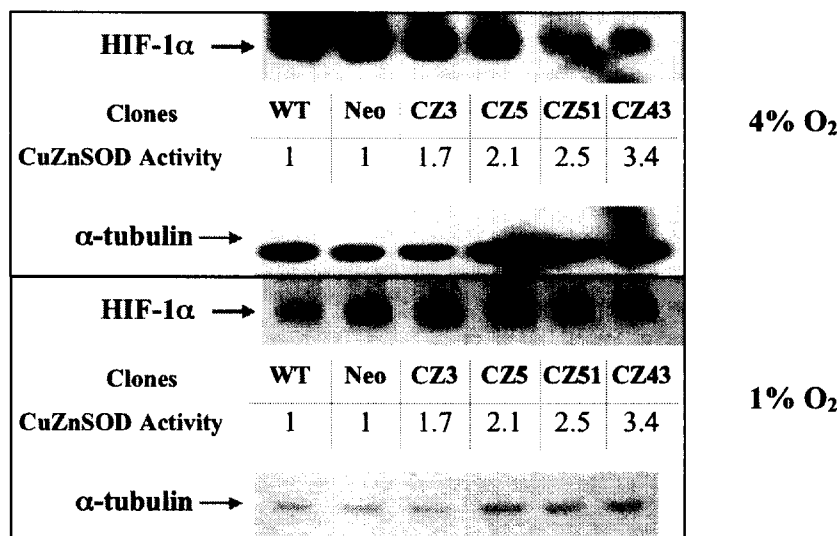
Figure. HIF-1α protein accumulation was suppressed by CuZnSOD in a dose-dependent manner at 4% O₂, but not at 1% O₂. Four different U118-9 clones stably transfected with human CuZnSOD cDNA (CZ3, CZ5, CZ51, CZ43), together with WT and Neo cells, were exposed to 4% O₂ (top panel) or 1% O₂ (bottom panel) for 4 h. After hypoxia exposure, cell lysates were collected and western blotting was carried out to measure HIF-1α protein levels.

Task 5:Prepare manuscripts and Ph.D. thesis.
----**Completed**

A manuscript has been submitted to Onogene, see attached. It has received a favorable review and we are currently revising it.

The PhD thesis has completed and submitted. I graduated with a PhD degree in July 2004.

Key Research Accomplishments

- Establishment of special protocols for HIF-1α protein western blotting.
  Specified in the Materials & Methods section of the attached manuscript: Protein harvest for HIF-1α western blot & HIF-1α western blot.
- Original findings on the regulatory effect of MnSOD on hypoxic accumulation of HIF-1α.
  We found that elevated MnSOD activity suppresses the hypoxic accumulation of HIF-1α protein. The suppression of MnSOD on HIF-1α protein accumulation was biphasic depending on MnSOD activity as summarized in the Body. This biphasic modulation can be observed under both 1% O_2 and 4% O_2 (Figure 1, 2 & 3).
- Findings on the regulatory effect of MnSOD on hypoxic induction of VEGF.
  Hypoxic induction of VEGF was also suppressed by elevated MnSOD activity and its expression levels reflected the protein levels of HIF-1α. (Figures 4 & 5 for VEGF mRNA levels and Figures 6 & 7 for VEGF protein levels in the appendix).
- Findings on the molecular mechanism by which MnSOD affects HIF-1α VEGF. We have determined that hydrogen peroxide appears to be a minor player in this effect and all indirect evidence points to a key role for superoxide.

Reportable Outcomes

Abstracts/Presentations:

 Wang MV, Zhang HJ, Kirk JS, et al. HIF-1 induction under hypoxia is modulated by MnSOD *FREE RADICAL BIOLOGY AND MEDICINE* 33: 424 Suppl. 2 2002

 M. Wang, H.J. Zhang, J.S. Kirk, L.W. Oberley, G.R. Buettner. (2002) Modulating HIF: Potential targets for cancer therapy. Poster at 2nd International conference on prostate cancer research, Iowa City, IA, October 12-15.

Fellowship based on this award:

 Graduate Incentive Fellowship, 2002, The University of Iowa.

Conclusions

The study is the first to demonstrate that not only dioxygen, but also the antioxidant enzyme MnSOD, which modulates the levels of superoxide and hydrogen peroxide, can determine the level of HIF-1α, and consequently the expression of VEGF, an essential factor for the growth of new blood vessels. This understanding of the control of HIF-1α and subsequent induction of VEGF points to new interventions that can enhance recovery from stroke, heart attack, trama as well as suggests new avenues for improved cancer therapy.

To be able to apply the presented research results to clinical studies, a better understand the regulatory mechanism is necessary. Therefore, we proposed to further pursue the molecular mechanisms controlling the biphasic suppressive effect of MnSOD on HIF-1α protein. Since one of the products of MnSOD enzymatic activity is hydrogen peroxide, which can diffuse across membranes freely, we examined the potential role of hydrogen peroxide as a key mediator of the suppressive effect of MnSOD. To address this question, the role of peroxide removing enzymes (catalase and glutathione peroxidase) was tested. We found that they produced only a minor effect on HIF-1α activation. Indirect evidence points to superoxide as a key species in the regulation of HIF-1α. Further experiments are in progress to better test the role of superoxide in HIF-1α activation.

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Paper

    M. Wang, J.S. Kirk, F.E. Domann, H.J. Zhang, F.Q. Schafer, C.J. Weydert, D.R. Spitz, G.R. Buettner & L.W. Oberley. (2004) Manganese superoxide dismutase suppresses hypoxic induction of hypoxia inducible factor-1α and vascular endothelial growth factor. *Oncogene*, in revision.

Abstracts

    M.V. Wang, H.J. Zhang, J.S. Kirk, L.W. Oberley, and G.R. Buettner. (2002) HIF-1 induction under hypoxia is modulated by MnSOD. *Free Radic. Biol. Med.* **33:** S444.

    M. Wang, H.J. Zhang, J.S. Kirk, Y. Zhang, L.W. Oberley, and G.R. Buettner. (2001) A connection between HIF-1 and SOD: Potential targets for cancer therapy. *Free Radic. Biol. Med.* **31:** S63.

    H. Duan, **M. Wang**, L.W. Oberley, and F.E. Domann. (2001) HIF-1alpha regulates promoter activity of the tumor suppressor gene maspin in human breast cancer cells. *Free Radic. Biol. Med.* **31:** S138.

Thesis

    Min Wang. (2004) Manganese superoxide dismutase suppressed the accumulation of hypoxia inducible fadtor-alpha and the induction of vascular endothelial growth factor under hypoxia. Ph.D. Thesis, The University of Iowa.

Personnel receiving pay from the research effort

Min Wang

Manganese superoxide dismutase suppresses hypoxic induction of hypoxia inducible factor-1α and vascular endothelial growth factor

Min Wang, Jeanie S. Kirk, Frederick E. Domann, Hannah J. Zhang, Sujatha Venkataraman, Freya Q. Schafer, Shawn Flanagan, Christine J. Weydert, Douglas R. Spitz, Garry R. Buettner & Larry W. Oberley

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Running title: MnSOD suppresses HIF-1 and VEGF

Key words: HIF-1, VEGF, angiogenesis, hypoxia, MnSOD.

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Abstract

Hypoxia inducible factor-1 (HIF-1) is a transcription factor that governs cellular responses to reduced O_2 availability by mediating crucial homeostatic processes. HIF-1 is composed of a HIF-1α subunit and a HIF-1β subunit. HIF-1α is degraded *via* enzyme-dependent hydroxylation of prolines of HIF-1α in the presence of molecular oxygen, Fe^{2+}, α-ketoglutarate, and ascorbate. These cofactors contribute to the redox environment of cells. The antioxidant enzyme manganese superoxide dismutase (MnSOD) also modulates the cellular redox environment. Here we show that MnSOD suppressed hypoxic accumulation of HIF-1α protein in human breast carcinoma MCF-7 cells. This suppression was biphasic depending on MnSOD activity. At low levels of MnSOD activity, HIF-1α protein accumulated under hypoxic conditions. At moderate levels of MnSOD activity (2- to 6-fold increase compared to parent cells), these accumulations were blocked. However, at higher levels of MnSOD activity (>6-fold increase), accumulation of HIF-1α protein was again observed. This biphasic modulation was observed under both 1% O_2 and 4% O_2. Co-expression of mitochondrial hydrogen peroxide removing proteins prevented the accumulation of HIF-1α protein in cells with high levels of MnSOD; this effect demonstrates that the restabilization of HIF-1α observed in high MnSOD overexpressors is probably due to hydrogen peroxide, most likely produced from MnSOD. Hypoxic induction of vascular endothelial growth factor (VEGF) protein was also suppressed by elevated MnSOD activity and its levels reflected HIF-1α protein levels. These observations demonstrated that HIF-1α accumulation could be modulated by the antioxidant enzyme MnSOD.

Introduction

Hypoxia describes a low oxygen concentration environment that can be caused by stroke, coronary artery disease, trauma, or high rate of cell proliferation such as encountered in solid tumors (Dewhirst et al., 1989). Because the proliferation rate of tumor cells is usually faster than that of normal endothelial cells that form microvessels into tumors, tumor microvessels do not function as efficiently as those in normal tissues, leading to the widespread hypoxia in solid tumors (Shah-Yukich & Nelson, 1988).

Tumor cells that have adapted to hypoxic conditions are thought to play critical roles in tumor progression (Hockel et al., 1996). Among the first responses at the onset of hypoxia is an increase in the protein levels of hypoxia-inducible factor-1 (HIF-1) (Wang & Semenza, 1993b). HIF-1 responds to reduced O_2 availability by mediating crucial homeostatic processes such as angiogenesis, glycolysis, and erythropoiesis (Semenza, 2000). As a transcription factor, HIF-1 has more than 40 target genes and the known number continues to increase. Vascular endothelial cell growth factor (VEGF) is one of HIF-1 downstream genes (Liu et al., 1995). It is a powerful mitogenic cytokine specific for endothelial cells; its production triggers the angiogenic cascade and tumor neovascularization processes (Nagy et al., 2002). VEGF controls not only the onset, but also the extent and duration of these processes. Hypoxia, which is a common characteristic of solid tumors, is a stimulus leading to the induction of VEGF *via* the regulation of HIF-1 (Kim et al., 2003; Pugh & Ratcliffe, 2003a; Semenza, 2001b).

HIF-1 is a heterodimer consisting of two subunits, HIF-1α and HIF-1β (Wang & Semenza, 1993a). When oxygen is sufficient, HIF-1α is constantly being made and then degraded by the ubiquitin-proteosome pathway *via* the von Hippel-Lindau (VHL) tumor suppressor protein (Maxwell et al., 1999). VHL protein binds to HIF-1α, causing ubiquitinylation of this protein, which is then degraded by the 26S proteosome (Salceda & Caro, 1997). The binding of VHL to

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HIF-1α requires hydroxylation of proline residues inside the oxygen-dependent degradation domain (ODD) of HIF-1α (Bruick & McKnight, 2001). Proline[402] and proline[564] are hydroxylated by HIF-prolyl hydroxylase, which needs the presence of several cofactors to gain full activity. These cofactors are: molecular oxygen, Fe^{2+}, α-ketoglutarate, and ascorbate (Jaakkola et al., 2001; Kondo & Kaelin, 2001; Pugh & Ratcliffe, 2003b; Semenza, 2001a). During hydroxylation, one atom of the O_2 molecule is incorporated into succinate upon decarboxylation of α-ketoglutarate and the other into a hydroxyl group on the proline residue. The Fe^{2+} is located inside the active site of HIF-prolyl hydroxylase (McNeill et al., 2002).

Reactive oxygen species (ROS) such as superoxide and hydrogen peroxide are known to serve as signal transducers (Nakamura et al., 1997; Powis et al., 1997; Schafer & Buettner, 2001; Suzuki et al., 1997). It has been shown that ROS can participate in the hypoxia signal transduction pathway that mediates the stabilization of HIF-1α (Chandel et al., 2000; Park et al., 2003; Schroedl et al., 2002). Manganese superoxide dismutase (MnSOD) is a primary antioxidant enzyme that is located in the mitochondrial matrix. It has been shown that the malignant phenotype is suppressed when MnSOD activity is elevated in certain cancer cells (Li et al., 1998a; Li et al., 1998b; Zhang et al., 1999). MnSOD has been implicated as a tumor suppressor and as a metastasis suppressor in some tumor cell lines (Bravard et al., 1992; Oberley & Buettner, 1979; Oberley & Oberley, 1988). MnSOD modulates the cellular redox environment by converting superoxide radical ($O_2^{\cdot-}$) to hydrogen peroxide (H_2O_2) and dioxygen. Therefore, MnSOD may affect the expression of redox-sensitive genes, including HIF-1. Here we are the first to show that MnSOD overexpression suppressed the hypoxic accumulation of HIF-1α in human breast carcinoma MCF-7 cells. The hypoxic induction of VEGF protein were also suppressed by elevation of MnSOD.

Results

Increased MnSOD activity suppressed hypoxic accumulation of HIF-1α protein in cells exposed to 1% O_2

To test the role of elevated MnSOD activity on hypoxic accumulation of HIF-1α protein, twelve previously characterized (Zhang et al., 1999) stably transfected MCF-7 clones with different MnSOD activities as well as parental (untransfected) cells were used. These clones were produced by transfection with pcDNA3 plasmids containing sense MnSOD cDNA or containing no MnSOD insert (Neo clone) by the lipofectAmine (Life Technologies, Gaithersburg, MD) method. The G418-resistant colonies were isolated by cloning rings and maintained in medium supplemented with 400 µg/ml G418 (Life Technologies). The MnSOD clones were characterized and found to have a less malignant phenotype than the parental cells as indicated by: 1) decreased plating efficiency; 2) elongated cell population doubling time; 3) lower clonogenic fraction in soft agar; 4) complete inhibition or delayed onset of tumor formation in nude mice, as well as slower growth of tumors that formed. These 13 different cell lines were exposed to 1% O_2 for 4 h. This condition did not change MnSOD activity during this time frame (data not shown). Immediately after hypoxia, protein was harvested and HIF-1α protein levels were analyzed by western blotti (**Figure 1a**). The results showed that at low levels of MnSOD activity (parental cells (WT) and transfected clones with the same activity (Neo and SOD23)), HIF-1α protein accumulated. In clones where MnSOD activity was moderately increased (2- to 6-fold relative to that of the parent cells), HIF-1α protein levels were decreased. The levels of HIF-1α protein decreased with increasing MnSOD activity until they were no longer detectable in clones with 3- and 6-fold increase in MnSOD activity. However, in clones where MnSOD activity was increased to a higher level (> 6-fold), HIF-1α protein was again detected, and increased with increasing MnSOD activity. Regression analysis demonstrated a linear inverse correlation of HIF-1α with

low levels of MnSOD activity (1- to 3- fold), whereas at high levels of MnSOD (> 6-fold), a linear positive correlation was observed (**Figure 1b**).

In order to confirm and extend this observation with another technique besides the stable transfection approach, MCF-7 cells were transduced with adenovirus-containing MnSOD cDNA. This transduction increased the enzymatic activity of MnSOD as determined by activity gel analyses; MnSOD activity increased with increasing MOI (multiplicity of infection, which is calculated from PFU, plaque forming units, **Figure 2a**). The increase in MnSOD activity with increasing MOI was confirmed with a spectrophotometric enzymatic activity assay (data not shown). The transduction efficiency after adenoviral infection using MnSOD immunofluorescence was also measured. A small percentage of the cells stained for MnSOD in the control cells (**Table 1**) and the percentage of cells stained increased with increasing MOI. At the maximum MOI used (200), 73% of the cells stained for MnSOD protein. Following adenovirus transduction in 21% O_2, cells were exposed to 1% O_2 for 4 h and samples were then collected. SOD activity (activity gel assay) and HIF-1α protein levels (western blotting) were assessed. In MCF-7 parental cells, HIF-1α protein accumulated after hypoxia exposure; increased MnSOD activity suppressed the accumulation of HIF-1α protein under 1% O_2 (**Figure 2b**). HIF-1α protein levels dropped significantly starting at 5 MOI of adenoviral MnSOD and became undetectable at 50 MOI. HIF-1α protein was again detectable at 75 MOI and increased with increasing MOI thereafter, but not reaching the level found in parental cells. This is consistent with what we observed with MCF-7 clones that were stably transfected with MnSOD cDNA, **Figure 1**. In both plasmid transfection and adenovirus transduction experiments, HIF-1α protein was undetectable in cells grown at 21% O_2 (data not shown).

Increased MnSOD activity suppressed hypoxia-induced accumulation of HIF-1α protein in cells exposed to 4% O$_2$

The oxygen concentration inside a large solid tumor is heterogeneous. Some regions have very low pO$_2$ values while others have pO$_2$ values near to that of normal tissues (Collingridge et al., 1997; Moulder & Martin, 1984). Because MnSOD suppressed HIF-1α protein accumulation in MCF-7 cells exposed to 1% O$_2$, both with adenoviral transduction and plasmid transfection, it was further investigated if this suppressive effect existed when cells were exposed to 4% O$_2$. This is a value typical of normal tissue oxygen concentration (Jiang et al., 1996) but still lower than the 21% O$_2$ usually used for most cell culture conditions. Using the same stable MnSOD-overexpressing clones described above, it was found that HIF-1α protein began to decrease in clones that expressed a 2-fold increase in MnSOD activity and remained suppressed in clones that had up to a 6-fold increase in MnSOD activity. However, when MnSOD activity was increased higher than 8-fold, HIF-1α levels were again increased (**Figure 3a**). In order to examine this with another method, MCF-7 cells transduced at 21% O$_2$ with increasing MOI of adenoviral MnSOD were then exposed to 4% O$_2$. As MnSOD activity increased with increasing adenovirus titer from that of parental MCF-7 cells, HIF-1α levels decreased, reaching a minimum at 20 and 50 MOI (**Figure 3b**). At 75 MOI, HIF-1α levels increased slightly and continued to increase with increasing MOI, but again not reaching the level of parental cells. The results observed at 4% O$_2$ parallel those of 1% O$_2$, suggesting that the suppressive effect of MnSOD on HIF-1α is common at both oxygen concentrations.

Hypoxia (1% O$_2$) induced VEGF mRNA expression in a time-dependent manner in MCF-7 cells

Angiogenesis is a key step that not only supports tumor growth with nutrients and oxygen, but also provides a ready route for cells to escape the primary site, leading to metastases (Brown

et al., 1997). Vascular endothelial growth factor (VEGF) is a potent angiogenic mitogen that mediates the angiogenesis process (Dvorak et al., 1995a; Nagy et al., 2002). Exposure to hypoxia stimulates the production of VEGF, mainly through the induction of the transcription factor HIF-1 (Kim et al., 2003). With the observation that MnSOD suppressed hypoxic accumulation of HIF-1α protein, the effect of increasing MnSOD activity on hypoxic induction of VEGF in MCF-7 cells was also measured.

WT MCF-7 cells after hypoxic exposure (1% O_2) for various times were lysed and total RNA isolated to be used in both semiquantitative and real time RT-PCR measurements. VEGF-A mRNA expression increased dramatically after cells were exposed to hypoxia for 6 h, compared to cells without hypoxia exposure. This induction continued in cells that were exposed for longer times (**Figure 4a**). To confirm these results, quantitative real-time RT-PCR for VEGF-A expression was also carried out using the primers described in Materials and Methods (**Figure 4b**). The results also suggested that VEGF-A expression increased with time of exposure of cells to 1% O_2. Therefore, real-time RT-PCR confirmed that VEGF-A expression increased in MCF-7 Wt cells after exposure to 1% O_2.

Hypoxic induction of VEGF-A mRNA was suppressed by increased MnSOD in cells exposed to 1% O_2 in stable transfectants

As VEGF-A mRNA expression increased substantially with 6 h of hypoxia, the effect of increased MnSOD activity on VEGF-A mRNA expression at this time was studied. Because MnSOD modulated HIF-1α and HIF-1α accumulation leads to VEGF expression, it was anticipated that MnSOD would modulate VEGF. Indeed, MnSOD transfection modulated VEGF-A expression with hypoxic exposure parallel to the observation with HIF-1α. After cells were exposed to 6 h hypoxia, VEGF-A mRNA expression was induced in parental cells. In cells with intermediate levels of MnSOD, the induction was suppressed (**Figure 4c**). To validate these

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findings, quantitative real-time RT-PCR was carried out with some of the clones that were subjected to hypoxia (1% O_2) and compared to that at 21% O_2 (**Figure 4d**). The VEGF-A expression in WT cells was more than 3-fold increased after 6 h of hypoxia compared to cells under 21% O_2. VEGF expression increased less than 1.5-fold in clone SOD 50 that has a 3-fold increase in MnSOD activity. The clone that had a 6-fold in MnSOD activity (Mn52) showed about a 2.5-fold increase VEGF-A mRNA levels. In the clone with higher MnSOD activity (19-fold, Mn11) the VEGF-A expression was suppressed in hypoxia, but not to the level observed in SOD50 clones. This data shows that VEGF-A mRNA levels are induced by hypoxia, but the levels are suppressed in MnSOD overexpressing cells with again a biphasic effect.

MnSOD suppressed the secretion of VEGF protein with hypoxia stimulation (1% O_2)

As a mitogenic cytokine, VEGF protein is usually secreted outside the cells to stimulate the proliferation of endothelial cells (Dvorak et al., 1995b). To determine if VEGF protein secretion followed the trend of its mRNA expression, the pattern of VEGF protein secretion with hypoxia stimulation was examined. MCF-7 WT cells were exposed to hypoxia for various times and VEGF concentrations were measured. Hypoxia exposure increased the amount of VEGF protein (**Figure 5a**). The amount of VEGF protein increased rapidly from 8 until 12 h in the hypoxia-treated cells; after 12 h, VEGF levels kept increasing, but at a lower rate than earlier times. VEGF protein also increased in non-hypoxic cells, but at a much slower rate than in hypoxic cells. Moreover, most of the VEGF protein accumulated in the medium and only small amounts were found inside the cells (**Figure 5a**).

To determine the suppressive effect of elevated MnSOD activity on the secretion of VEGF protein, seven stably transfected MCF-7 clones with various MnSOD activity (described above) together with parental cells were exposed to hypoxia (1% O_2) for 12 hours. Medium from these clones were then collected and VEGF concentrations were measured. Increased MnSOD

activity significantly decreased VEGF protein levels (**Figure 5b**). The suppression of VEGF protein by MnSOD was biphasic, with the greatest suppression at low to medium MnSOD levels and less suppression at higher MnSOD levels, consistent with what was observed with HIF-1α protein and VEGF mRNA levels.

Adenoviral transduction of cellular catalase (cCAT) increased catalase activity in MCF-7 Neo vector control cells

Throughout this work, a biphasic effect was observed. One possible explanation for the stabilization of HIF-1α seen at high levels of MnSOD is that hydrogen peroxide produced from MnSOD overwhelms the endogenous peroxide removing systems and causes HIF-1α to increase. In order to examine this hypothesis, extracellular hydrogen peroxide levels in these cell lines stably overexpressing MnSOD were measured. Cells overexpressing MnSOD did have higher levels of exogenous H_2O_2, but there was no difference between hypoxic and non-hypoxic cells (data not shown). This result suggested that hydrogen peroxide could be the effector of the biphasic effect since it was elevated in cells with high levels of MnSOD. Moreover, we have shown in a previous paper that in the MCF-7 clones the levels of intracellular hydrogen peroxide as measured by DCF-DA fluorescence increased as the levels of MnSOD increased (Zhang et al., 2002). If the effects seen at high levels of MnSOD are due to the measured increased levels of hydrogen peroxide, then peroxide removal would be expected to eliminate the biphasic effect and prevent HIF-1α stabilization. Catalase (CAT) is one of the most intensively studied H_2O_2 removing enzymes. Therefore, it was decided to examine the effect of increasing catalase activities with the adenoviral transduction technique in MnSOD overexpressing cells.

We first determined whether adenoviral transduction of human cellular catalase (cCAT) could increase catalase activity in MCF-7 cells, and at the same time, determined a proper dose for further experiments. Adenoviral catalase cDNA was introduced into MCF-7 Neo vector control

cells at increasing MOI (calculated from plaque forming units). Catalase activities were determined by running catalase activity gels. Catalase activities increased with increasing MOI of adenoviral catalase (**Figure 6a**). A dose of 100 MOI of adenoviral catalase was used for further experiments.

Adenoviral transduction or inhibition of native cellular catalase (cCAT) did not alter the MnSOD suppressive effect on HIF-1α protein accumulation

Human cellular catalase (cCAT, 100 MOI) was introduced into MnSOD stably transfected MCF-7 clones. Besides MCF-7 WT and Neo, three other clones were used in these experiments: SOD50 with a 3-fold increase in MnSOD activity compared to MCF-7 WT cells, Mn52 with a 6-fold increase in MnSOD activity, and Mn11 with a 19-fold increase in MnSOD activity. We first showed that neither catalase protein nor activity was significantly different in the various lines (data not shown); moreover, catalase protein levels were not changed by hypoxia (data not shown). Cells were incubated with adenovirus carrying human catalase for 24 h. Adenovirus was removed and fresh medium was added onto cells for another 24 h. Cells were then exposed to hypoxia for 4 h to induce HIF-1α. Western blotting was used to detect HIF-1α protein levels in these clones. Without catalase transduction, these five clones showed the biphasic suppressive effect of increased MnSOD expression on HIF-1 induction (**Figure 7b**). That is, HIF-1α protein accumulated in MCF-7 WT and Neo clones with low MnSOD activity. In clones with medium MnSOD activity (SOD50 and Mn52), the accumulation of HIF-1α protein was suppressed, whereas in the clone with high MnSOD activity (Mn11), HIF-1α protein again accumulated. Contrary to what was expected, cCAT transduction did not cause significant changes in HIF-1α protein levels in any of these clones (**Figure 6b,c**). Increasing catalase activity by transducing catalase did not alter the suppressive effect of MnSOD.

Since MCF-7 cells have relatively high endogenous catalase activity, adding more catalase into the cells may not show any significant effect. Therefore, the effect of inhibiting endogenous catalase was next examined. 3-Amino-1,2,4-triazole (AT) is an irreversible inhibitor of catalase activity (Darr and Fridovich, 1986). Cells treated with AT showed a decrease in catalase activity (data not shown). In order to determine the role of endogenous catalase in mediating the suppressive effect of MnSOD, MCF-7 cells were treated with AT (18 mM, 24 h) to decrease endogenous catalase activity. Cells were then exposed to hypoxia (1% O_2) for 4 h. Western blotting was carried out to determine HIF-1α protein changes. Similar to increasing catalase activity with adenoviral transduction, catalase inhibition induced by AT did not cause significant changes in HIF-1α protein levels in MnSOD overexpressing clones (data not shown). These results suggested that peroxide removal by catalase did not play a role in mediating the suppressive effect of MnSOD on hypoxic induction of HIF-1. Since MnSOD is located in the mitochondria and catalase removes peroxide outside the mitochondria in either peroxisomes or cytoplasm, the location of the peroxide removing system may be important and responsible for these negative results.

Adenoviral transduction of mitochondrial catalase (mCAT) increased catalase activity in MCF-7 Neo vector control cells

Since neither native catalase transduction nor catalase inhibition changed the suppressive effect of MnSOD, a human adenoviral catalase construct with a mitochondrial targeting sequence was obtained to test whether the location of the peroxide removing system was important. Adenoviral mitochondrial catalase (mCAT) was made by Dr. Shawn Flanagan from a plasmid kindly given to us by Dr. Andres Melendez of Albany Medical College. The recombinant catalase cDNA contains an added mitochondrial-targeting sequence, but the peroxisomal targeting sequence has not been removed. Thus, the protein is expected to be found in both mitochondria and peroxisomes; this has been confirmed in published studies (Bai et al., 1999; Rodriguez et al., 2000).

It was first determined whether adenoviral transduction of mCAT could increase catalase activity in MCF-7 cells, and at the same time, a proper dose for further experiments was ascertained. Adenoviral mCAT cDNA was introduced into MCF-7 Neo vector control cells at increasing MOI (calculated from the plaque forming units). Catalase activity increased with adenovirus-mediated gene transfer (**Figure 7a**). High levels of catalase activity were achieved with as low as 20 MOI. A dose of 50 MOI of adenoviral mCAT was used for further experiments.

Adenoviral transduction of mitochondrial catalase (mCAT) decreased HIF-1α protein levels in a clone with high MnSOD activity

Adenoviral mCAT (50 MOI) was introduced into MnSOD stably transfected MCF-7 clones. In addition to MCF-7 WT and Neo, three other clones were used in these experiments: SOD50 with a 3-fold increase in MnSOD activity compared to MCF-7 WT cells, Mn52 with a 6-fold increase in MnSOD activity, and Mn11 with a 19-fold increase in MnSOD activity. Cells were incubated with adenovirus carrying mCAT for 24 h. Adenovirus was removed and fresh medium was added to cells for another 24 h. Cells were then exposed to hypoxia for 4 h to induce HIF-1α. Western blotting was used to detect HIF-1α protein levels in these clones.

In MCF-7 WT and Neo cells, the transduction of mCAT did not alter HIF-1α protein levels. In clones with medium MnSOD activity, clone SOD50 (3-fold) and clone Mn52 (6-fold), HIF-1α protein levels did not change with mCAT transduction (**Figure 7b**). Interestingly, HIF-1α protein levels decreased in clone Mn11 with mCAT transduction compared to the same cells without mCAT transduction (**Figure 7b,c**). Mn11 is the clone with high MnSOD activity (19-fold). Densitometric analysis showed that HIF-1α protein levels decreased significantly in Mn11 after the transduction of mCAT to a level of about 50% of the untransduced cells (**Figure 7c**).

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Adenoviral transduction of glutathione peroxidase–1 (GPx-1) increased GPx activity in MCF-7 Neo vector control cells

The transduction of native cellular catalase (cCAT) did not cause any change in HIF-1α protein levels in any of the clones, whereas the transduction of mitochondrial catalase (mCAT) decreased the HIF-1α protein levels in the clone with high MnSOD activity. These results indicate that the location of the peroxide-removing system is important to be able to alter the suppressive effect of overexpressed MnSOD. Therefore, the effect of transducing another peroxide removing enzyme, glutathione peroxidase-1 (GPx-1), was tested. An immunogold assay had previously verified that the adenoviral GPx-1 entered both cytosol and mitochondria (Li et al., 2000). It was first determined whether adenoviral transduction of GPx-1 could increase GPx activity in MCF-7 cells, and at the same time, a proper dose for further experiments was ascertained. Adenoviral GPx1 cDNA was introduced into MCF-7 Neo vector control cells at increasing MOI (calculated from the plaque forming units). GPx activity increased with adenovirus-mediated gene transfer (**Figure 8a**). Because high levels of catalase activity were achieved with 100 MOI, a dose of 100 MOI of adenoviral GPx was used for further experiments.

Adenoviral transduction of GPx-1 decreased HIF-1α protein levels in a clone with high MnSOD activity

Adenoviral GPx-1 (100 MOI) was introduced into MnSOD stably transfected MCF-7 clones. Besides MCF-7 WT and Neo, three other clones were used in these experiments: SOD50 with a 3-fold increase in MnSOD activity compared to MCF-7 WT cells, Mn52 with a 6-fold increase in MnSOD activity, and Mn11 with a 19-fold increase in MnSOD activity. All of these lines had non-detectable GPx activity as measured by activity gels before transduction in cells grown in either 21% or 1% oxygen (data not shown). Cells were incubated with adenovirus

carrying GPx-1 for 24 h. Adenovirus was removed and fresh medium was added onto cells for another 24 h. Cells were then exposed to hypoxia for 4 h to induce HIF-1α. Western blotting was used to detect HIF-1α protein levels in these clones.

In MCF-7 WT and Neo cells, the transduction of GPx-1 did not alter HIF-1α protein levels. In clones with medium MnSOD activity, clone SOD50 (3-fold) and clone Mn52 (6-fold), HIF-1α protein levels did not change with GPx-1 transduction (**Figure 8b**). Similar to what was observed with mitochondrial catalase (mCAT) transduction, HIF-1α protein levels decreased in clone Mn11 after the adenoviral infection of GPx-1 (**Figure 8b**). Mn11 is the clone with high MnSOD activity (19-fold). Densitometric analysis showed that HIF-1α protein levels decreased significantly in Mn11 after the transduction of GPx-1 to a level about 75% less than untransduced cells (**Figure 8c**).

Discussion

Hypoxia is a deficiency in oxygen (Ozaki et al., 1999; Semenza et al., 1999). Hypoxia is widespread in solid tumors due to an inefficient vascular supply of oxygen. Hypoxia-inducible factor-1 (HIF-1) is an important transcription factor that is activated in conditions of decreased oxygen (Semenza et al., 1998). HIF-1 mediates cell survival in hypoxia by promoting genes involved in glucose homeostasis, erythropoiesis, and angiogenesis (Semenza, 1999). While normal tissue and benign breast tumors do not exhibit increased HIF-1 activity, evidence for a graded increase in activity has been demonstrated in the progression from pre-neoplastic lesions to cancer metastases (Bos et al., 2001). Moreover, a positive correlation has been found between HIF-1 expression and vascularization in brain tumors (Zagzag et al., 2000).

As one of the HIF-1 downstream genes, VEGF is a pivotal mitogen that mediates endothelial cell proliferation and new vessel formation, a process known as angiogenesis. Hypoxic induction of VEGF is mediated by hypoxia inducible factor-1 (Forsythe et al., 1996).

MnSOD is an important antioxidant enzyme involved in cancer cell growth. A change in the level of MnSOD enzyme activity will change the redox status of the cell and affect the expression of redox-sensitive genes and proteins. HIF-1 is known to be a redox-sensitive protein, but the effect of MnSOD on HIF-1 expression has not been studied until the present work (Chandel et al., 2000; Huang et al., 1996; Kelley & Parsons, 2001). It has been shown both *in vitro* and *in vivo* that tumor growth is suppressed with MnSOD overexpression (Li et al., 1998a; Li et al., 1998b; Zhang et al., 1999). MnSOD has been proposed as a tumor suppressor gene (Bravard et al., 1992; Oberley & Oberley, 1988). It catalyzes the reaction: $2O_2^{\cdot-} + 2H^+ \rightarrow H_2O_2 + O_2$. It is well accepted that a change in MnSOD activity will result in a change in the steady-state level of superoxide ($O_2^{\cdot-}$). That a moderate increase in MnSOD decreased the accumulation of HIF-1α suggests an involvement of superoxide. It has been observed that a moderate increase in nitric oxide ($NO^{\cdot}$) decreased HIF-1α accumulation (Mateo et al., 2003; Thomas et al., 2004). Because $NO^{\cdot}$ reacts rapidly with $O_2^{\cdot-}$, an increase in $NO^{\cdot}$ will decrease the steady-state level of $O_2^{\cdot-}$, similar to an increase in MnSOD activity. These observations suggest that superoxide could modulate HIF-1α accumulation.

However, a product of MnSOD enzymatic activity, hydrogen peroxide (H_2O_2), is a highly diffusible molecule that moves freely across cell membranes. H_2O_2 also contributes to the redox environment of the cell (Oberley & Buettner, 1979). Therefore, H_2O_2 may also be a link between MnSOD and HIF-1α. Both superoxide and hydrogen peroxide could react with the iron of HIF-prolyl hydroxylase, modulating its activity and affecting the accumulation of HIF-1α. However, superoxide in general causes reduction of iron, while hydrogen peroxide will oxidize it:

$$Fe^{3+} + O_2^{\cdot-} \rightarrow Fe^{2+} + O_2.$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + HO^{\cdot}$$
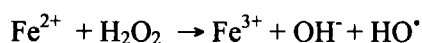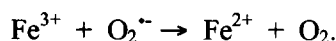
If Fe^{2+} is necessary for HIF-1α-prolyl hydroxylase (PH) activity, then superoxide radical should increase PH activity, and hydrogen peroxide should decrease PH activity. Thus, superoxide radical should decrease the levels of HIF-1α, while hydrogen peroxide should increase the levels of HIF-1α. Our data as discussed below show just this for hydrogen peroxide, but the opposite for superoxide radical: the higher the SOD levels and thus the lower the levels of superoxide radical, the lower the levels of HIF-1α, until very high levels of SOD are reached.

We have shown here that MnSOD suppressed hypoxic accumulation of HIF-1α protein, which led to lower levels of VEGF. Moderate levels of MnSOD activity (2- to 6-fold increase compared to parent cells) in MCF-7 cells abolished the hypoxic accumulation of HIF-1α protein and the induction of VEGF. Surprisingly, higher levels of MnSOD allowed HIF-1α protein accumulation, showing a biphasic modulation on HIF-1α accumulation. Interestingly, in these high MnSOD overexpressing cells, HIF-1α never returned to values near those of the parent cells; in other words, HIF-1α was still suppressed relative to parent cells and vector control. We hypothesize that elevation of MnSOD to moderate levels (physiological levels for most normal tissues, see Oberley et al., 1989) will lower the steady-state level of superoxide, which then lowers HIF-1α levels. However, if MnSOD is increased above these levels, H_2O_2 could be a modulator. An amount of peroxide may be produced that is above the levels that the endogenous peroxide-removing capabilities of the cell can accommodate. Too much hydrogen peroxide then leads to inhibition of HIF-1α degradation and HIF-1α accumulates. As mentioned above, this effect of high MnSOD could be due to oxidation of iron, removing the required Fe^{2+} and also producing the damaging hydroxyl radical.

It is well accepted that increasing SOD levels lowers the steady-state levels of superoxide radicals, but there is still controversy over whether the levels of hydrogen peroxide increase (Li et al., 2000; Omar and McCord, 1990). We have argued that in cells, hydrogen peroxide levels must

increase after increases in MnSOD based on three observations: 1) after stable overexpression of MnSOD, in many clones either CAT or GPx levels are found to increase also (Li et al., 1998; Liu et al., 1997; Zhong et al., 1997); 2) stable expression of either CAT (Rodriquez et al., 2000) or GPx (Li et al., 2000) modulates the effects of MnSOD overexpression; 3) assays of either intracellular (Zhang et al., 2002) or extracellular hydrogen peroxide (Wenk et al., 1999) concentrations show increases after MnSOD overexpression. The problem with the measurements of hydrogen peroxide is that the extracellular measurements are quite specific, but it unclear where the hydrogen peroxide is coming from, and the intracellular measurements are done with non-specific assays. We have measured both intracellular and extracellular hydrogen peroxide in the cells used in the present work. Using dihydrofluorescein diacetate (DFH-DA), we found that fluorescence increased in the stable MnSOD-overexpressing MCF-7 cell lines as the MnSOD activity increased (Zhang et al., 2002). The fluorescence was most likely due to hydrogen peroxide since it was blocked by adenoviral CAT or GPx and by the GPx mimic ebselen (Zhang et al., 2002). Moreover, in the present study, we measured extracellular hydrogen peroxide in these cell lines and found that the levels increased with increasing MnSOD activity (data not shown). Thus, the levels of both intracellular and extracellular hydrogen peroxide appear to increase as the MnSOD levels increase. The reason why hydrogen peroxide levels increase after MnSOD overexpression is still the subject of intense study.

Another possibility for the effecter of MnSOD overexpression is molecular oxygen. MnSOD produces both H_2O_2 and O_2. Moreover, reduction of Fe^{3+} by superoxide also produces O_2 as shown above. HIF-1α degradation ensues upon the hydroxylation of prolines as accomplished by HIF prolyl hydroxylase. This specific prolyl hydroxylase requires 2-oxoglutarate, Fe^{2+}, ascorbate, and molecular oxygen for enzymatic activity (Jaakkola et al., 2001; Kondo & Kaelin, 2001; Pugh & Ratcliffe, 2003b; Semenza, 2001a). All of these cofactors can be

found in mitochondria as well as cytoplasm. Thus, the oxygen produced by MnSOD is also a possible effecter for the MnSOD-suppressive effect we have observed.

Our results are consistent with what we have observed with tumor incidence and tumor growth in nude mice after MnSOD overexpression (Zhang et al., 1999). That is, the incidence of tumors and growth of the tumors that do form decreases when MnSOD activity is increased to a moderate level (<6-fold). However, MnSOD does not further suppress the tumor formation when its activity is increased to a higher level (>6-fold), and indeed some of the tumor-suppression effect is lost (Zhang et al., 1999). This suggests that the *in vivo* tumor suppressive function of MnSOD may be achieved by the inhibition of HIF-1 protein accumulation. By controlling HIF-1 protein and thereby controlling the expression of HIF-1 downstream genes that are involved in tumor metabolism and growth, such as VEGF, MnSOD may control a switch for genes that allow tumor growth and progression. These results suggest that MnSOD may inhibit tumor angiogenesis and that part of the tumor suppressive effect seen *in vivo* may be due to inhibition of angiogenesis.

In support of this proposal, Wheeler *et al.* have shown that adenovirus transduction of SOD3 (extracellular SOD or ecSOD) inhibits the growth of B16 melanoma cells in mice (Wheeler et al., 2003). Two weeks after implantation, B16 tumor size was 65% smaller in mice infected with Ad*ecSOD* in comparison with mice infected with Ad*lacZ*. Tumors from Ad*ecSOD*-infected mice expressed less VEGF protein. Importantly, blood vessel density as assessed by two different ways was markedly reduced in tumors from Ad*ecSOD*-infected mice compared with controls. Wheeler *et al.* did not examine the effects of MnSOD overexpression and did not determine if HIF-1 was involved. However, this study is consistent with our findings of the dramatic effect of MnSOD overexpression on HIF-1α accumulation and VEGF expression.

Last, a strong inducer of MnSOD is TNF-α (Wong et al., 1989). Therefore, one would expect that TNF-α would induce MnSOD in certain cells, leading to lowered levels of HIF-1α and VEGF. Future work is needed to investigate the role of cytokines in the regulation of this pathway.

Materials and Methods

Cell culture. Human breast adenocarcinoma MCF-7 cells were routinely cultured in Eagles's MEM containing 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, and 10% FBS, and incubated at 37°C with 95% air and 5% CO_2. MCF-7 clones that were stably transfected with MnSOD and Neo were cultured in the same medium supplemented with 400 µg/mL G418 (Life Technologies, Inc.). Medium was generally changed every 3-4 days.

Hypoxic treatment. Cells were seeded into 60 mm tissue culture dishes (Corning Scientific Products DIV, Acton, MA) and medium was replaced with fresh medium before hypoxic treatment. The dishes were transferred to a modular incubator chamber (Billups-Rothenberg, Del Mar, CA) that was flushed with either 1% O_2 (1% O_2, 5% CO_2 and balanced with N_2) or 4% O_2 (4% O_2, 5% CO_2 and balanced with N_2) for 4 min at a rate of 20 L/min, then sealed and placed at 37°C.

Adenovirus infection. Adenoviral MnSOD, adenoviral cCAT, and adenoviral GPx were made originally in the laboratory of Dr. John Engelhardt (Zwacka et al., 1998; Li et al., 2001) and were manufactured at The University of Iowa's Vector Core Facility or Viraquest, Inc. The original AdGPx produced a product with a myc tag (Li et al., 2001), but in the present work the AdGPx was modified so that it produced a native GPx without a myc tag. The mitochondrial catalase plasmid was obtained from Dr. Andres Melendez and was made into an adenovirus construct by Viraquest, Inc. with the help of Dr. Shawn Flanagan. For western and activity gel analyses, MCF-7 cells were plated into 60 mm dishes at a density of 5×10^5 cells/plate in full media. The next day adenovirus (prepared in 3% sucrose/PBS) was added to the cells containing 2 mL of media in each dish. To the appropriate dishes, 1.25 - 200 multiplicity of infection (MOI) of adenoviral constructs were added. The MOI for all experiments was calculated from the plaque forming units, which was determined by the Vector Core or Viraquest, Inc. Following a 24 h incubation, the viral particles

were removed and fresh medium was added. All infections were done at 21% O_2. Exposure to 1% O_2 and 4% O_2 was done at 24 hours after adenoviral removal.

Adenoviral Transduction Efficiency. MCF-7 cells (2×10^4) were plated into 8-well slides (Nunc) and allowed to attach overnight. Various titers of Ad*MnSOD* (1.25-200 MOI) or Ad*LacZ* (200 MOI) were added for 24 h. Media was changed at 24 h. Cells were fixed with 4% gluteraldehyde at 48 h. Cells were incubated with human MnSOD primary antibody and goat anti-rabbit Alexa 488 secondary antibody (Molecular Probes). The cell images were captured and analyzed with Image J. Five hundred cells were analyzed for each MOI from 3 to 4 fields.

Cell homogenization for SOD activity gel and SOD western blot. Cells were washed in phosphate-buffered saline (PBS: KCl 2.7 mM, KH_2PO_4 1.5 mM, $NaHPO_4$ 8 mM and NaCl 136.9 mM, pH 7.0), scrape harvested, and pelleted at 12,000 x g for 10 s in 1.5 mL microfuge tubes. The supernatant was removed, cells were resuspended in 50 mM phosphate buffer (pH 7.8) and sonicated on ice for 3 x 30 s using a Vibra Cell cup horn sonicator (Sonics and Materials, Inc., Danbury, CT) at maximum power. Protein concentration was estimated by the Bradford method (Biorad Laboratories, Hercules, CA) and standardized with bovine serum albumin.

Antioxidant protein activity gels. In this technique, non-dissociating electrophoresis gels are run (Tulchin et al., 1976) with ammonium persulfate used as the initiator in the running gel (12.5%) and riboflavin-light in the stacking gel (5%). Once run, the gels were stained for SOD activity by the method of Beauchamp and Fridovich (Beauchamp & Fridovich, 1971). The gels were stained for CAT or GPx activity by the method of Sun et al. (Sun et al., 1988).

MnSOD activity assay

SOD activities were determined using the modified NBT method described earlier (Oberley and Spitz, 1984; Spitz and Oberley, 1989)). The competition reaction between SOD and the superoxide

indicator molecule, nitroblue tetrazolium (NBT), is the basis of this indirect assay. Xanthine/xanthine oxidase were used to generate superoxide, the substrate of SOD. Various amount of total protein sample was added to the reaction until the maximal inhibition of the reaction was obtained as measured by a spectrophotometer at 560 nm. Total SOD activity was determined by the amount of protein necessary for half-maximal inhibition of the NBT reaction. MnSOD activity was quantified in the presence of 5 mM sodium cyanide, which inhibits CuZnSOD activity. One unit of activity was defined as the concentration of SOD that reduced the NBT reaction to one-half of the maximum.

SOD western blot. The amount of immunoreactive MnSOD protein was measured by western blotting (Oberley et al., 1989). Briefly, cell homogenates (30 μg) were processed by SDS-PAGE and transferred to PVDF (Millipore Corporation, Bedford, MA) membranes. The membrane was then probed with rabbit anti-MnSOD IgG (1: 1000 dilution). The secondary antibody was goat anti-rabbit IgG (Pharmingen/Transduction Laboratories, San Diego, CA) used at 1:5000 dilution. Blots were visualized using Chemiluminescent Developer (Pierce, Rockford, IL) and exposed to film. The bands were examined with a computerized digital imaging system using AlphaImager 2000 software (Alpha Innotech, San Leandro, CA). The IDV (integrated density values) were obtained by integrating all of the pixel values in the area of one band after correction for background.

Protein harvest for HIF-1α western blot. Medium was removed from tissue culture dishes. After rinsing twice with PBS, 100 μL boiling lysis buffer (1% SDS, 1.0 mM sodium ortho-vanadate, 10 mM Tris pH 7.4) was added to the cells. Cell lysates were then scraped and transferred into micro-centrifuge tubes and boiled for 5 min. Protein concentrations were determined with Bio-Rad DC protein assay (BioRad Laboratories, Hercules, CA) as described by the manufacturer.

HIF-1α western blot. 30 μg total protein was separated on a 4-20% gradient Tris-HCl polyacrylamide ready gel (BioRad Laboratories, Hercules, CA). Then the protein was electrotransferred onto a PVDF membrane (Millipore Corporation, Bedford, MA) by running at 100 V for 1 h. For HIF-1α western blot, the primary antibody was mouse anti-HIF-1α IgG (Pharmingen/Transduction Laboratories, San Diego, CA) used at 1:2000 dilution. For α-tubulin western blot, the primary antibody was mouse anti-human α-tubulin (Santa Cruz Biotechnology, Santa Cruz, CA) used at 1:5000 dilution. The secondary antibody used against both primary antibodies was goat anti-mouse IgG (Pharmingen/Transduction Laboratories, San Diego, CA) used at 1:2000 dilution. Blots were visualized using Chemiluminescent Developer (Pierce, Rockford, IL) and exposed to film. The bands were examined with a computerized digital imaging system using AlphaImager 2000 software (Alpha Innotech, San Leandro, CA). The IDV (integrated density values) were obtained by integrating all of the pixel values in the area of one band after correction for background.

Semiquantitative RT-PCR analysis. Total RNA was extracted by Qiagen RNeasy kit (Qiagen Inc., Valencia, CA) as recommended by the manufacturer. The RT-PCR was carried out using a Qiagen One-Step RT-PCR kit (Qiagen, Valencia, CA) according to the manufacturer's recommendation. The RT-PCR product was electrophoresed on a 1% agarose gel.

Analysis of VEGF-A expression using quantitative real-time RT-PCR. The isolated RNA was reverse transcribed using cDNA High-Capacity Archive kit according to the manufacturer's protocol (Applied Biosystems, Foster City, California). qRT-PCR was performed on an ABI PRISM 7000 (Applied Biosystems, Foster city, CA) using the TaqMan PCR master mix (Applied Biosystems, Foster city, CA) . Primers for amplification of VEGF-A cDNA were designed using Primer Express (ABI) and were: 5′-CTC TAC CTC CAC CAT GCC AAG-3′ (forward) and 5′-AGA CAT CCA TGA ACT TCA CCA CTT C-3′ (reverse) and were obtained from IDT Inc

(Coralville, IA). The PCR reactions were performed in 50 μL volumes containing 150 ng cDNA, 270 nM of forward and reverse primers for VEGF-A, 0.09 μM fluorescent VEGF-A probe (IDT Inc.) and 2x- TaqMan PCR master mix. The PCR sequential thermal cycling profile was as follows; 95°C for 10 min followed by 40 cycles of 95 °C for 15 s with a final extension of 60°C for 1 h. Since amplification products were detected using TaqMan, dissociation curves were performed to verify specificity of the products formed during PCR (data not shown). 18S rRNA was measured using the SYBR Green PCR master mix (ABI) and the expression level of VEGF-A was normalized to 18S rRNA.

Statistics. Pearson's regression and correlation analysis were used to determine the relationship between the MnSOD activities and HIF-1α protein levels. The R^2 and p-value was calculated using Microsoft Excel software.

Abbreviations: HIF-1: hypoxia inducible factor-1; MnSOD: manganese superoxide dismutase; MOI: multiplicity of infection; NBT, nitroblue tetrazolium; ROS: reactive oxygen species; VEGF: vascular endothelial growth factor; VHL: von Hippel-Lindau.

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Table 1

Ad*MnSOD* transduction increased immunofluorescence in MCF-7 cells by 70% with 200 MOI. 500 cells from 3-4 fields were counted.

MOI	Cells Stained (%)
0	9
Ad*LacZ* (200 MOI)	2
1.25	22
2.5	25
5	23
20	36
50	47
75	45
100	58
200	73

Legend to Figure 1

Plasmid transfection of MnSOD suppressed HIF-1α protein accumulation after 4 h exposure to 1% O_2. **a.** Western blot showing HIF-1α protein levels in 12 different MnSOD stably transfected clones plus parental MCF-7 cells after 4 h exposure to 1% O_2. The first 3 lanes were samples from: MCF-7 parental cells, Neo vector control, and a clone with the same activity as parental cells. The remaining 10 lanes were samples from clones with increasing MnSOD activity. The bottom row of numbers indicates the relative MnSOD activity compared to the parental MCF-7 cells. The actual MnSOD activities of the clones were previously measured (Zhang et al., 1999) to be (in units/mg protein): WT (7±2), Neo4 (7± 3), SOD15 (19± 4), SOD18 (11±2), SOD23 (10±2), SOD50 (23±6), Mn1 (62±8), Mn11 (142±20), Mn28 (57±10), Mn44 (44±9), Mn52 (44±6), Mn59 (53±2), Mn63 (52±8).

b. Regression analysis shows a biphasic effect. HIF-1α protein levels were quantified by densitometry and normalized to α-tubulin levels. Similar results were obtained in a repeated experiment.

Legend to Figure 2

Adenoviral transduction of MnSOD suppressed HIF-1α protein accumulation after 4 h exposure to 1% O_2. **a.** MnSOD activity gel analysis showed that MnSOD activity increased with increasing MOI (from 1.25 up to 200). Adenoviral transduction was performed at 21% O_2 and the transduced cells were exposed to 1% O_2 for 4 h. Samples labeled LacZ were from cells transduced with Ad*LacZ*. **b.** Western blots showed HIF-1α protein levels in Ad*MnSOD* transduced cells exposed to 1% O_2 for 4 h. α-Tubulin was used as an internal control. Primary antibodies to HIF-1α and α-tubulin were mixed together to probe the blot. Similar results were obtained in a repeated experiment.

Legend to Figure 3

Elevated MnSOD activities suppressed HIF-1α protein accumulation at 4% O_2. **a.** Plasmid transfection of MnSOD suppressed normoxic (4% O_2, 4 h) accumulation of HIF-1α protein. Western blot shows HIF-1α protein levels in different MnSOD stably transfected clones of MCF-7 cells with 4 h of exposure to 4% O_2. The top row of numbers indicates the relative MnSOD activity in 12 different clones compared to MCF-7 WT cells. α-Tubulin was used as an internal control. The 12 different clones are the same as used in **Figure 1**. Similar results were obtained in repeated experiments. **b.** Adenoviral transduction of MnSOD suppressed HIF-1α protein accumulation at 4% O_2. The top panel shows that MnSOD activity increased with increasing MOI (from 1.25 up to 200). Adenoviral transduction was performed at 21% O_2 and the transduced cells were exposed to 4% O_2. The second and third panels shows western blots for HIF-1α and α-tubulin, respectively, from cells exposed to 4% O_2 for 4 h. Similar results were obtained in repeat experiments.

Legend to Figure 4

MnSOD overexpression suppressed hypoxic induction of VEGF-A mRNA in MCF-7 cells. **a.** Steady-state levels of VEGF mRNA increased with time when MCF-7 WT cells were subjected to hypoxia. Cells were exposed to hypoxia (1% O_2) for various times. Total RNA was then isolated and semiquantitative RT-PCR was carried out. β-Actin was used as the internal control. Similar results were observed in repeated experiments. M: DNA marker; N: negative control (without mRNA samples); 0, 2, 4, 6, 12, 24 h are different times of hypoxia exposure (1% O_2). **b.** Quantitative RT-PCR analysis also documented an increase in VEGF-A expression with time (0 to 24 h) of exposure of MCF-7 Wt cells to 1% hypoxia. The VEGF-A expressions in samples were normalized with 18S rRNA. Values are mean ± SE of three samples each from two separate experiments. * group statistically different from 0 time controls at $p < 0.05$ level. **n=3 c.** A representative result shows that MnSOD overexpression suppressed hypoxic (1% O_2, 6 h) induction of VEGF mRNA in 7 distinct MnSOD stably transfected clones as well as parental MCF-7 cells. M: DNA marker; N: negative control (without mRNA samples). **d.** Quantitative RT-PCR analysis of VEGF-A expression in different MnSOD overexpressing clones subjected to 6 h of hypoxiacompared to cells grown at 21% (0 h of hypoxia) is shown. The bar graph illustrates that there is differential induction of VEGF-A expression in MCF-7 clones. *group statistically different from 0 h control group at $p < 0.05$ level. **n=3**

Legend to Figure 5

MnSOD overexpression suppressed VEGF protein secretion in MCF-7 cells. **a.** Hypoxia induced the secretion of VEGF protein in a time-dependent manner in MCF-7 WT cells. MCF-7 WT cells were exposed to hypoxia (1% O_2) for various times. VEGF concentration was measured with a Human VEGF-ELISA kit (R&D Systems, Minneapolis, MN). VEGF production increased when cells were exposed to hypoxia for longer times. VEGF was mainly secreted outside the cell as the concentration of VEGF in tissue culture medium was much higher than that in cell lysate. Values represent mean ± S.D of three measurements of a single sample. **b.** MnSOD overexpression suppressed VEGF protein secretion in MCF-7 cells. VEGF protein was measured after 12 h of hypoxia (1% O_2) in 7 distinct clones plus MCF-7 parental cells with various MnSOD activities. Values represent mean ± S.E.M. of three independent experiments. *hypoxia-treated cells were different from the non-hypoxic cells at the $p<0.05$ level.

a. Adenoviral transduction of peroxisomal/cytosolic catalase (cCAT) increased catalase activities in MCF-7 Neo vector control cells. Cells (1×10^6) were seeded in 100 mm^2 tissue culture dishes. Adenoviral cCAT was added onto the cells at various MOI that was calculated from PFU (plaque forming units). After 24 h of infection, medium containing adenovirus was removed and fresh medium was added onto the cells. Cells were then allowed to recover for 24 h. After recovery, cells were scraped and centrifuged to obtain a cell pellet. Cell lysates were then obtained by sonication. Catalase activity gel assay was carried out. Thirty µg protein per lane was used in this experiment. Experiment was repeated with similar results. **b.** Adenoviral transduction of human catalase (cCAT) did not alter the MnSOD suppressive effect on HIF-1α protein accumulation. Human catalase (cCAT, 100 MOI) was introduced into five MnSOD stably transfected MCF-7 clones: MCF-7 WT and Neo, SOD50 (3-fold increase in MnSOD activity compared to MCF-7 WT), Mn52 (6-fold increase in MnSOD activity), and Mn11 (19-fold increase in MnSOD activity). Cells were incubated with adenovirus carrying cCAT for 24 h. Medium containing adenovirus was removed and fresh medium was added onto cells for another 24 h. Cells were then exposed to hypoxia for 4 h to induce HIF-1α. Western blot was used to detect HIF-1α protein levels in these clones. α-tubulin was used as an internal control. **c.** Densitometric analysis of the western blot. Values represent mean ± S.D. n=3. No group was statistically different with transduction vs. without transduction.

Legend to Figure 7

a. Adenoviral transduction of mitochondrial catalase (mCAT) increased catalase activities in MCF-7 Neo vector control cells. Cells (1×10^6) were seeded in 100 mm^2 tissue culture dishes. Adenoviral mCAT was added onto the cells at various MOI, which was calculated from PFU (plaque forming units). After 24 h of infection, medium containing adenovirus was removed and fresh medium was added onto the cells. Cells were then allowed to recover for 24 h. After 24 h of recovery, cells were scraped and centrifuged to obtain a cell pellet. Cell lysates were then obtained by sonication. Catalase activity gel assay was carried out. Thirty μg protein per lane was used in this experiment. Experiment was repeated with similar results. **b.** Adenoviral transduction of mitochondrial catalase (mCAT) decreased HIF-1α protein levels in clone with high MnSOD activity. Mitochondrial catalase (mCAT, 50 MOI) was introduced into five of the MnSOD stably transfected MCF-7 clones: MCF-7 WT and Neo, SOD50 (3-fold increase in MnSOD activity compared to MCF-7 WT), Mn52 (6-fold increase in MnSOD activity), and Mn11 (19-fold increase in MnSOD activity). Cells were incubated with adenovirus carrying mCAT for 24 h. Medium containing adenovirus was removed and fresh medium was added onto cells for another 24 h. Cells were then exposed to hypoxia for 4 h to induce HIF-1. . Western blot was used to detect HIF-1α protein levels in these clones. α-tubulin was used as an internal control. HeLa cell lysate was used as HIF-1α protein positive control. **c.** Densitometric analysis of the western blot. Values represent mean ± S.D. of results from three independent samples. No transduced group was found to be statistically different from the untransduced group.

Legend to Figure 8

a. Adenoviral transduction of GPx-1 increased GPx activity in MCF-7 Neo vector control cells. Cells (1×10^6) were seeded in 100 mm^2 tissue culture dishes. After cells were attached, adenoviral GPx-1 (100 MOI) was added onto the cells at various MOI that was calculated from PFU (plaque forming units). After 24 h of infection, medium containing adenovirus was removed and fresh medium was added onto the cells. Cells were then allowed to recover for 24 h. Then, cells were scraped and centrifuged to obtain cell pellets. Cell lysates were then obtained by sonication. GPx activity gel assay was then carried out (150 μg protein per lane was used in this experiment). Experiment was repeated with similar results. **b.** Adenoviral transduction of glutathione peroxidase-1 (GPx-1) decreased HIF-1α protein levels in clone with high MnSOD activity. Adenoviral GPx-1 (100 MOI) was introduced into five of MnSOD stably transfected MCF-7 clones: MCF-7 WT and Neo, SOD50 (3-fold increase in MnSOD activity compared to MCF-7 WT), Mn52 (6-fold increase in MnSOD activity), and Mn11 (19-fold increase in MnSOD activity). Cells were incubated with adenovirus carrying mCAT for 24 h. Medium containing adenovirus was removed and fresh medium was added onto cells for another 24 h. Cells were then exposed to hypoxia for 4 h to induce HIF-1α. Western blot was used to detect HIF-1α protein levels in these clones. α-Tubulin was used as an internal control. **c.** Densitometric analysis of the western blot. Values represent mean ± S.D. of results from three independent samples. *GPx-treated group was statistically different from the untreated group at the $p < 0.05$ level.

Figure 1.

A.

HIF-1α

Clones: Wt | Neo | SOD23 | SOD18 | SOD15 | SOD50 | Mn44 | Mn52 | Mn59 | Mn63 | Mn28 | Mn1 | Mn11

MnSOD Activity: 1 | 1 | 1 | 2 | 3 | 3 | 6 | 6 | 7 | 7 | 8 | 9 | 19

α-tubulin

B.

$R^2 = 0.98$
$p < 0.001$

$R^2 = 0.78$
$p = 0.02$

HIF-1α / tubulin

Relative MnSOD activity (-fold)

Figure 2.

A.

MOI	0	LacZ	1.25	2.5	5	20	50	75	100	200

MnSOD

B.

HIF-1α

MOI	0	LacZ	1.25	2.5	5	20	50	75	100	200

α-tubulin

Figure 3.

A.

| MnSOD activity | 1 | 1 | 1 | 2 | 3 | 3 | 6 | 6 | 7 | 7 | 8 | 9 | 19 |

HIF-1α

α-tubulin

B.

| MOI | 0 | LacZ | 1.25 | 2.5 | 5 | 20 | 50 | 75 | 100 | 200 |

MnSOD

HIF-1α

α-tubulin

Figure 4.

A.

hypoxia (h)   M   N   0   2   4   6   12   24

600 bp ⟶   ⟵ β-actin
                                                  ⟵ VEGF

B.

C.

	non-hypoxia		6 h hypoxia	MnSOD activity (relative)
M N	1 1 2 3 3 6 7 19	1 1 2 3 3 6 7 19	

⟵ β-actin
⟵ VEGF

1   5   10   15   19
                      lane

D.

Figure 5

Figure 6

A.

Catalase

MOI   0   LacZ   20   50   100   200   300

B.

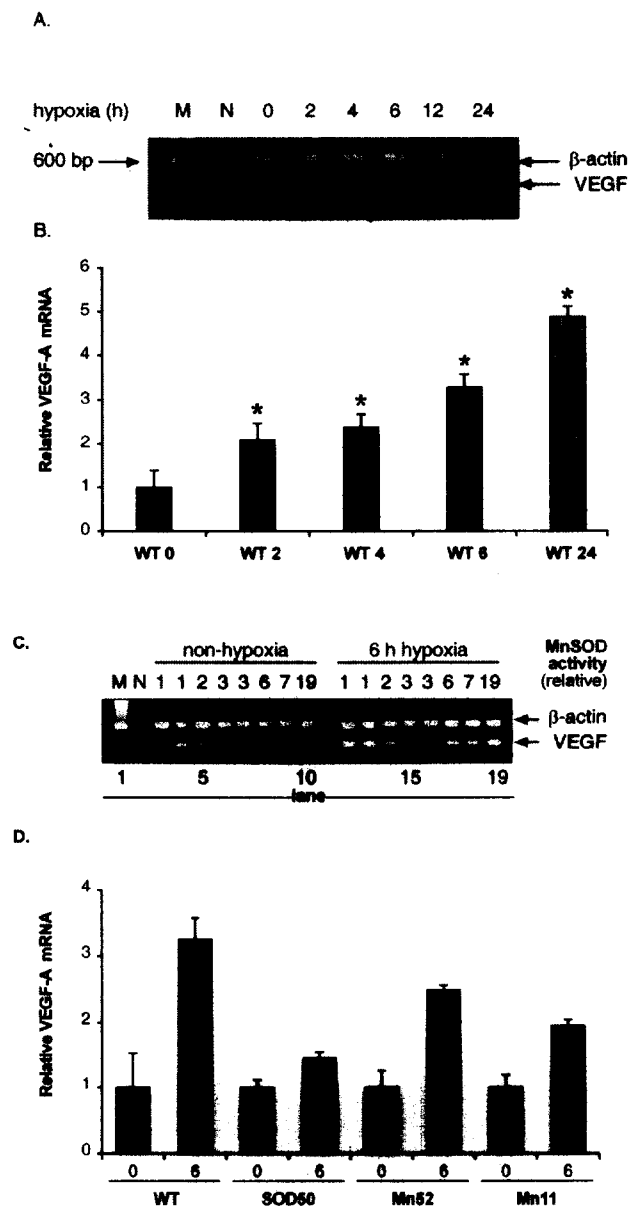| | – 21 % O_2 – | | | | 1 % O_2 | | | |
c CAT	–	+	–	+	–	+	–	+	–	+	–	+
HIF-1α												
clone	WT		WT		Neo		SOD50		Mn52		Mn11	
MnSOD activity (fold)	1		1		1		3		6		19	
α-tubulin												

C.

Figure 7

A.

Catalase							
AdmCAT MOI	0	LacZ	20	50	100	200	300

B.

_____ 1 % O₂ _____

mCAT   −   +   −   +   −   +   −   +   −   +

HIF-1α

clone	WT	Neo	SOD50	Mn52	Mn11
MnSOD activity (fold)	1	1	3	6	19

α-tubulin

C.

■ No mCAT
□ mCAT

Clone names (Relative MnSOD activity/fold)

47

Figure 8

A.

	GPx								
MOI	STD		0	LacZ	20	50	100	200	300

B.

— 21 % O$_2$ — ————————— 1 % O$_2$ —————————

GPx	−	+	−	+	−	+	−	+	−	+	−	+
HIF-1α												
clone	WT		WT		Neo		SOD50		Mn52		Mn11	
MnSOD activity (fold)	1		1		1		3		6		19	
α-tubulin												

C.

■ No GPx
□ GPx

HIF-1α/tubulin

Clone names (Relative MnSOD activity/fold)

WT (1x)   Neo (1x)   SOD50 (3x)   Mn52 (6x)   Mn11 (19x)